Caffeine Inhibition of Sterigmatocystin, Citrinin, and Patulin Production

ROBERT L. BUCHANAN, MOLLY A. HARRY, and MICHAEL A. GEALT

ABSTRACT

Microbiological medium was used to study the effect of caffeine on sterigmatocystin, citrinin, and patulin production by *Aspergillus versicolor*, *Penicillium citrinum*, and *Penicillium urticae*, respectively. Caffeine inhibited the growth of the three fungal species and decreased sterigmatocystin and patulin production. The effect on citrinin production appeared to be limited to a delay in toxin synthesis. A preliminary examination of *P. urticae* suggested that caffeine's anti-mycotoxigenic activity does not involve a generalized inhibition of lipid synthesis.

INTRODUCTION

PREVIOUS INVESTIGATIONS have established that while cocoa and coffee beans are commonly contaminated with aspergilli (Hansen and Welty, 1970; Hiscocks, 1965), they are generally poor substrates for aflatoxin production (Levi et al., 1975; Nartowicz et al., 1979; Lenovich and Hurst, 1979; Llwewllyn et al., 1978). Nartowicz et al. (1979) and Lenovich (1981) attributed this anti-aflatoxigenic activity to the caffeine content of these commodities. It has also been established that the addition of caffeine to microbiological media at levels ⩾1 mg/ml inhibits aflatoxin production (Buchanan and Fletcher, 1978), while theophylline (Buchanan and Fletcher, 1978) and theobromine (Buchanan et al., 1978) have little activity. Similar results have been reported for ochratoxin A production by *Aspergillus ochraceus* (Buchanan et al., 1982). Buchanan et al. (1982) found that caffeine inhibited the growth of a number of mycotoxigenic *Aspergillus* and *Penicillium* species, and suggested that caffeine may have inhibitory activity against the production of a variety of polyketide mycotoxins. The objective of the present study was to characterize further caffeine's anti-myotoxigenic activity by determining its effect on the production of three additional polyketide mycotoxins; sterigmatocystin, citrinin, and patulin.

MATERIALS & METHODS

Microorganisms

Aspergillus versicolor NRRL 5219, *Penicillium citrinum* NRRL 5927, and *Penicillium urticae* (*P. patulum*) NRRL 994 were used to study sterigmatocystin, citrinin, and patulin production, respectively. Stock cultures were maintained on potato dextrose agar (Difco) slants stored at 4°C. Spore suspensions were prepared as previously described (Tice and Buchanan, 1982), and diluted to contain approximately 10^6 conidia/ml.

Medium

YES medium (6% sucrose + 2% yeast extract) (Davis et al., 1966) supplemented with caffeine (0, 0.5, 1, 2, or 3 mg/ml) was used throughout the study.

Author Buchanan is affiliated with the USDA-ARS, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, PA 19118. Author Harry is affiliated with the Dept. of Nutrition & Food Sciences, and Author Gealt is with the Dept. of Biological Sciences, Drexel Univ., Philadelphia, PA 19104.

Culture techniques

The effect of caffeine on mycotoxin production was studied by transferring 50 ml portions of medium supplemented with the appropriate caffeine level to 250-ml Erlenmeyer flasks, which were then capped and autoclaved for 10 min at 15 psi. The flasks were inoculated with 1.0 ml of spore suspension to achieve an inoculum level of approximately 4×10^3 conidia/ml. All flasks were then incubated without agitation at 28°C. The effect of caffeine on lipid production was studied in a similar manner except that 500-ml flasks containing 100 ml of medium were employed.

Mycotoxin analyses

After 3, 7, 10, and 14 days, triplicate cultures of each caffeine level were extracted three times with 50 ml of chloroform (sterigmatocystin and citinin) or ethyl acetate (patulin). The extracts from each culture were pooled, concentrated to 10 ml using a flash evaporator, and stored at −20°C while awaiting analysis. After extraction, the mycelia were collected on preweighed filter paper (Whatman #1), rinsed with distilled water, and dried in an 85°C oven for 18 hr. Mycelial dry weights were then determined gravimetrically.

Sterigmatocystin was separated by the AOAC-recommended thin-layer chromatographic (TLC) technique (Horwitz, 1975), and quantitated using a fluorodensitometer. Citrinin was separated by the TLC method outlined by Hald and Krogh (1973), followed by fluorodensitometric quantitation. Patulin production was quantitated using a modification of the high pressure liquid chromatographic technique described by Moller and Josefsson (1980). The ethyl acetate extracts were evaporated to dryness under N_2, and redissolved in water. Pautlin samples were then separated on a reverse phase column (C_{18}, 5μ, 25 cm) eluted with water/methanol (9:1, 1 ml/min). Patulin was detected by UV absorption at 282 nm, and quantitated by calculating peak areas, and comparing against patulin standards.

Lipid analysis

Mycelia from *P. urticae* cultures containing 0, 0.5, and 2 mg caffeine/ml were collected by filtration, washed, and extracted with 150 ml of methanol/chloroform (2:1), followed by 150 ml of methanol chloroform/water (2:1:0.8) (Folch et al., 1957). The extracts were combined, 60 ml each of chloroform and water were added, and the solvent layers allowed to separate. The chloroform layers of each caffeine level were collected and pooled, and the solvent removed with a flask evaporator. The lipid was redissolved in 15 ml of diethyl ether, filtered to remove precipitated caffeine, and washed three times with 5 ml of water. The extracts were then transferred to preweighed vials, the solvent removed under N_2, and the total lipid content estimated gravimetrically.

Major lipid classes were estimated by TLC analysis using Silica gel G-60 plates (20 X 20 cm). Samples were spotted along with an ergosterol + lanosterol + cholesteryl standard, and eluted with benzene/ethyl acetate (80:20). After elution, the bands corresponding to desmethylsterols, dimethylsterols, and sterol esters + triacylglycerols (these two components co-chromatographed) were extracted from the silica gel twice with chloroform and once with diethyl ether. The dissolved lipid fractions were transferred to individual preweighed vials, evaporated under N_2, and the lipid component estimated gravimetrically.

RESULTS & DISCUSSION

THE EFFECTS OF CAFFEINE on growth and sterigmatocystin production by *A. versicolor* are depicted in Table 1. Caffeine did not appear to affect the growth of the mold during the first 3 days, but concentrations ⩾1 mg/ml in-

Table 1—Growth and sterigmotocystin production by Aspergillus versicolor cultured in YES medium containing various levels of caffeine

Caffeine (mg/ml)	Incubation time (days)	Mycelium[a] (mg)	Sterigmatocystin (μg/culture)	Sterigmatocystin mycelium (μg/g)
0.0	3	270 ± 40[b]	ND[c]	–
	7	790 ± 20	44.6 ± 7.5[d]	57
	10	1300 ± 110	253.3 ± 44.6	195
	14	1310 ± 10	354.2 ± 27.5	270
0.5	3	280 ± 30	ND	–
	7	610 ± 40	0.8 ± 0.1	1
	10	1220 ± 30	158.8 ± 65.7	130
	14	1340 ± 30	455.6 ± 16.5	340
1.0	3	240 ± 30	ND	–
	7	350 ± 20	ND	–
	10	560 ± 30	ND	–
	14	890 ± 90	14.3 ± 7.9	16
2.0	3	270 ± 10	ND	–
	7	320 ± 10	ND	–
	10	370 ± 10	ND	–
	14	440 ± 10	ND	–
3.0	3	330 ± 50	ND	–
	7	270 ± 20	ND	–
	10	290 ± 20	ND	–
	14	400 ± 10	ND	–

[a] Mycelium dry weight
[b] Mean + standard deviation of three cultures
[c] None detected
[d] Mean + standard deviation of triplicate samples, each assayed in duplicate

Table 2—Growth and citrinin production by Penicillium citrinum cultured in YES medium containing various levels of caffeine

Caffeine (mg/ml)	Incubation time (days)	Mycelium[a] (mg)	Citrinin (mg/culture)	Citrinin mycelium[a] (mg/g)
0.0	3	470 ± 80[b]	1.12 ± 0.85[c]	2.38
	7	1310 ± 10	5.70 ± 0.49	4.35
	10	1020 ± 90	2.53 ± 0.54	2.48
	14	770 ± 40	1.83 ± 0.39	2.38
0.5	3	320 ± 40	0.03 ± 0.02	0.09
	7	1100 ± 40	4.11 ± 0.35	3.74
	10	990 ± 90	2.66 ± 0.28	2.68
	14	780 ± 20	1.69 ± 0.05	2.17
1.0	3	250 ± 20	ND[d]	–
	7	630 ± 70	2.06 ± 0.70	3.27
	10	1110 ± 50	8.20 ± 0.31	7.39
	14	840 ± 20	1.50 ± 0.16	1.79
2.0	3	310 ± 10	ND	–
	7	370 ± 10	ND	–
	10	410 ± 40	4.08 ± 0.47	9.95
	14	570 ± 100	5.43 ± 0.13	9.53
3.0	3	320 ± 10	ND	–
	7	370 ± 20	ND	–
	10	300 ± 60	ND	–
	14	420 ± 20	0.69 ± 0.09	1.64

[a] Mycelium dry weight
[b] Mean + standard deviation of triplicate cultures
[c] Mean + standard deviation of triplicate samples assayed in duplicate
[d] None detected

Table 3—Growth and patulin production by Penicillium urticae cultured in YES medium containing various levels of caffeine

Caffeine (mg/ml)	Incubation time (days)	Mycelium[a] (mg)	Patulin (μg/culture)	Patulin mycelium (μg/g)
0.0	3	250 ± 90[b]	436.8 ± 90.1[b]	1750
	7	1180 ± 90	436.1 ± 117.7	370
	10	1100 ± 50	4.6 ± 0.7	4
	14	1010 ± 90	3.5 ± 0.7	3
0.5	3	130 ± 20	16.5 ± 5.1	127
	7	1120 ± 40	196.9 ± 101.3	176
	10	1160 ± 20	5.4 ± 7.5	5
	14	1070 ± 20	0.8 ± 0.7	<1
1.0	3	100 ± 10	5.4 ± 1.6	54
	7	710 ± 90	27.2 ± 2.9	38
	10	1070 ± 30	38.6 ± 21.4	36
	14	980 ± 40	0.4 ± 0.6	<1
2.0	3	20 ± 2	2.7 ± 1.2	135
	7	270 ± 110	3.9 ± 1.8	14
	10	940 ± 100	97.0 ± 7.5	103
	14	1060 ± 40	5.1 ± 7.6	5
3.0	3	40 ± 30	ND[c]	–
	7	30 ± 30	ND	–
	10	420 ± 90	10.4 ± 5.3	25
	14	220 ± 60	4.4 ± 1.6	20

[a] Mycelium dry weight
[b] Mean + standard deviation of three replicates
[c] None detected

hibited further increases in mycelial dry weight. This pattern of growth inhibition was similar to that noted with other *Aspergillus* species (Buchanan et al., 1982). Sterigmatocystin production was inhibited to a greater extent than growth, with no toxin being detected in cultures containing ⩾2 mg caffeine/ml. Caffeine at 1 mg/ml delayed and decreased sterigmatocystin production, while 0.5 mg/ml had little effect. Again, this pattern of inhibition is similar to that previously noted for the production of aflatoxin (Buchanan and Fletcher, 1978) and ochratoxin A (Buchanan et al., 1982).

Caffeine's effect on growth and citrinin production by *P. citrinum* is summarized in Table 2. Growth was inhibited at caffeine levels ⩾1 mg/ml, with a pattern of inhibition similar to that observed with *A. versicolor*. However, caffeine's effects on citrinin production appeared to be primarily limited to a delay in toxin synthesis. In the 1 and 2 mg/ml cultures, an initial inhibition of citrinin production was followed by an apparent period of rapid "catch-up" synthesis, such that the levels of toxin produced ultimately reached or exceeded that of the control cultures. Whether this "catch-up" citrinin production would have occurred in the 3.0 mg/ml cultures if the incubation period had been extended is not known.

Caffeine at levels ⩾1 mg/ml inhibited or delayed growth of *P. urticae* (Table 3). However, this species appeared to be more resistant to caffeine, and even the 3 mg/ml level cultures had resumed increasing in dry weight by the 10th day of incubation. Patulin production was more strongly affected, with caffeine levels ⩾0.5 mg/ml producing decreased and delayed toxin production.

Comparison of the data from the present study along with that from previous investigations on aflatoxin and ochratoxin A production suggest that while caffeine can inhibit both growth (as measured by mycelial dry weight) and mycotoxin production, the two processes may not be directly related. For example, with *A. parasiticus, A. ochraceus*, and *A. versicolor*, both growth and toxin synthesis were strongly affected, while in *P. citrinum* growth was inhibited but anti-mycotoxigenic effects were limited primarily to delayed synthesis. Conversely, *P. urticae* was relatively resistant to growth inhibition, but patulin production was strongly suppressed by caffeine.

Patulin synthesis involves a multi-enzyme complex similar to, but distinctly separate from the fatty acid synthase complex (Scott et al., 1974). Likewise, the syntheses of other polyketide mycotoxins appear to involve a similar mechanism that is closely related to fatty acid synthesis. In the case of the aflatoxins, investigators (Detroy and Hesseltine, 1969; Shih and Marth, 1974) have suggested that there may be a relationship between mycotoxin pro-

Table 4—Effect of caffeine on accumulation of lipids by Penicillum urticae

Caffeine (mg/ml)	Day	Lipid/mycelium[a] mg/g Total	Desmethyl-sterols	Dimethyl-sterols	Triacylglycerols + sterol esters
0	3	111.4	20.2	16.5	45.8
	7	30.6	5.5	4.8	7.1
	10	45.1	3.5	1.0	1.2
0.5	3	158.2	19.8	23.2	47.2
	7	28.6	2.7	1.1	6.2
	10	45.6	2.7	1.3	0.8
2	3	141.1	ND[b]	3.6	12.9
	7	117.1	12.5	3.7	27.9
	10	80.0	2.1	ND	5.5

[a] Mycelial dry weight determined after extraction of lipids
[b] None detected

duction and total lipid synthesis. The similarities among polyketide and fatty acid syntheses suggested that caffeine might also affect lipid accumulation. Comparing mycelial dry weight with and without lipid extraction, Buchanan et al. (1982) hypothesized that caffeine may inhibit lipid synthesis in *A. ochraceus* and other mycotoxigenic fungi. To assess this hypothesis, preliminary experimentation was carried out to determine the effect of caffeine on the amount of lipid present within mycelia of *P. urticae*. The results observed for total lipid production and specific lipid components are presented in Table 4.

The concentration of total intracellular lipids in the control cultures was greatest after 3 days of incubation, and declined in the older cultures. Similarly, the levels of desmethylsterols, dimethylsterols, and triacylglycersol + sterol ester fractions were greatest in the young cultures and declined with age. Aside from a possible increase in total lipid content, the incorporation of 0.5 mg/ml caffeine into the medium had little effect on the level of lipids detected in *P. urticae*.

Increasing the concentration of caffeine to 2 mg/ml (an inhibitory concentration) did not affect total lipid accumulation in the 3-day samples, but did appear to depress desmethylsterol, dimethylsterol, and triacylglycerol + sterol ester accumulation. However, after 7 days of incubation, total lipids, desmethylsterols, and triacylglycerol + sterol ester levels appeared elevated, even though growth was strongly inhibited. Likewise, total lipid and triacylglycerol + sterol ester levels appeared elevated in the 10-day cultures. While additional research will be needed to confirm the results of this preliminary study, the data did not support the hypothesis that the inhibition of polyketide mycotoxin synthesis by caffeine involves a generalized inhibition of lipid synthesis.

The present study, in conjunction with previous investigations, indicates that caffeine can influence growth and polyketide mycotoxin production by a number of *Aspergillus* and *Penicillium* species. This suggests that the "function" of caffeine in commodities such as cocoa and coffee may be as a naturally-occurring fungistatic agent. The mechanism by which caffeine inhibits polyketide mycotoxin synthesis is unknown, and additional studies are currently underway to elucidate the compound's mode of action.

REFERENCES

Buchanan, R.L. and Fletcher, A.M. 1978. Methylxanthine inhibition of aflatoxin production. J. Food Sci. 43: 654.

Buchanan, R.L., Applebaum, R.S., and Conway, P. 1978. Effect of theobromine on growth and aflatoxin production by Aspergillus parasiticus. J. Food Safety 1: 211.

Buchanan, R.L., Tice, G., and Marino, D. 1982. Caffeine inhibition of ochratoxin A production. J. Food Sci. 47: 319.

Davis, N.D., Diener, U.L., and Eldridge, D.W. 1966. Production of aflatoxin B_1 and G_1 by Aspergillus flavus in a semi-synthetic medium. Appl. Microbiol. 14: 378.

Detroy, R.W. and Hesseltine, C.W. 1969. Net synthesis of ^{14}C-labeled lipids and aflatoxins in resting cells of Aspergillus parasiticus. Dev. Ind. Microbiol. 10: 127.

Folch, J., Lees, M., and Sloane-Stanley, G. 1957. A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 226: 497.

Hald, B. and Krough, P. 1973. Analysis and chemical confirmation of citrinin in barley. J. Assoc. Off. Anal. Chem. 56: 1440.

Hansen, A.P. and Welty, R.E. 1970. Microflora of raw cocoa beans. Mycopathol. Mycolog. Appl. 44: 309.

Hiscocks, E.S. 1965. The importance of mold in the deterioration of tropical foods and foodstuff. In "Mycotoxins in Foodstuff," (Ed.) G.N. Wogan. MIT Press, Cambridge, MA.

Horwitz, W. (Ed.). 1975. "Official Methods of Analysis of the Association of Official Analytical Chemists," 12th ed., p. 492. Washington, DC.

Lenovich, L.M. 1981. Effect of caffeine on aflatoxin production in cocoa beans. J. Food Sci. 46: 655.

Lenovich, L.M. and Hurst, W.J. 1979. Production of aflatoxin in cocoa beans. J. Assoc. Off. Anal. Chem. 62: 1076.

Levi, C.P., Trenk, H.L., and Yeransian, J.A. 1975. Investigations of mycotoxin relative to coffee. "7th International Colloquium on the Chemistry of Coffee," Association Scientifique International du Cafe, Paris.

Llwewllyn, G.C., Benevides, J., and Eadie, T. 1978. Differential production of aflatoxin on natural and heat-treated cocoa beans. J. Food Protect. 41: 785.

Moller, T.E. and Josefsson, E. 1980. Rapid high pressure liquid chromatography of patulin in apple juice. J. Assoc. Off. Anal. Chem. 63: 1055.

Nartowicz, V.B., Buchanan, R.L., and Segall, S. 1979. Aflatoxin production in regular and decaffeinated coffee beans. J. Food Sci. 44: 446.

Scott, A.I., Beadling, L.C., Georgopapadakou, N.H., and Subbarayan, C.R. 1974. Biosynthesis of polyketides. Purification and inhibition studies of 6-methylsalicylic acid synthase. Bioorganic Chem. 3: 238.

Shih, C.N. and Marth, E.H. 1974. Aflatoxin formation, lipid synthesis, and glucose metabolism by Aspergillus parasiticus during incubation with and without agitation. Biochem. Biophys. Res. Commun. 50: 237.

Tice, G. and Buchanan, R.L. 1982. Regulation of aflatoxin biosynthesis: Effect of exogeneously supplied cyclic nucleotides. J. Food Sci. 47: 153.

Ms received 11/1/82; revised 2/16/83; accepted 2/17/83.

Presented at the 42nd Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, June 22—26, 1982.

We thank Donald Lewis for his technical assistance.